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Prevalence of Human Papillomavirus, Cytomegalovirus, and Epstein-Barr Virus in the Cervix of Healthy Women

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The prevalence of some sexually transmitted viruses, possibly involved in cervical carcinogenesis, was studied in the cervix of women with normal cytology. The presence of human papillomaviruses (HPV) type 16 and 18, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) genomes in cervical cells taken from 143 healthy Italian women was investigated using the polymerase chain reaction (PCR). The study population was divided into four groups with respect to age as follows: group I, 17 to 25 years, n = 48 women; group II, 26 to 35 years, n = 30; group III, 36 to 50 years, n = 32; and group IV, 51 to 70 years, n = 33. In the first age group prevalence rates of HPV 16, CMV and EBV infection of 23%, 21% and 19% were found respectively. The infection rates of HPV 16 and CMV were shown to decrease with age, with prevalences of HPV 16 at 10% in the second group, 6% in the third and 3% in the fourth and of CMV at 13% in the second and third and 6% in the fourth groups. The prevalence of EBV infection did not decrease with increasing age (19% in the first and third groups, 20% in the second and 18% in the fourth). The occurrence of HPV 18 genome was very low (0–3%) and independent of age. In the first age group a higher percentage of double infections (16.6%) was found than in the three other age groups (6% in the second and third and 3% in the fourth). The finding of multiple infections in younger women requires further study in order to clarify the implications of such viral infections in healthy women and their contribution to the development of genital tract malignancies.

KEY WORDS: Epidemiology, normal cervix, sexually transmitted viruses

INTRODUCTION

Cervical cancer is one of the most important female malignancies worldwide and it is the second leading cause of cancer death in women in developed countries [Parkin et al., 1993; Boring et al., 1993]. The distribution of cervical cancer shows geographical differences with a very high frequency in Columbia and South America, where cervical cancer is the most common neoplasm in women, and low incidence in the female populations of the United States and Israel. Recent studies indicate that the European countries with the highest number of cervical neoplasms are Germany, Poland, Roumania and Hungary, whereas the lowest rates are found in Spain and Finland [Levi et al., 1989]. Italy is in a medium-low position [Zanetti and Crosignani, 1992].

Epidemiological observations indicate that cervical cancer is a sexually transmitted disease, but much controversy surrounds its aetiology. There is a body of evidence that cervical neoplasia is related to viral infection.

During the last few years a strong association has been demonstrated between infections with certain types of human papillomavirus (in particular HPV 16 and 18) and the development of invasive cervical carcinoma. HPV sequences have also been detected in a variety of other human epithelial tumours such as oral cancer [Yeudall, 1992], laryngeal carcinoma [Brandsma et al., 1986] and transitional tumours of the urinary bladder [Aglianò et al., 1994], but the evidence is not conclusive. Although HPVs may be important for cervical cancer development they are neither essential nor pose sufficient risk in themselves to cause cancer. Thus, a synergism has been postulated between two virus infections or between virus infection and initiating events in the carcinogenesis of cervical cancer [Zur Hausen, 1982; Herrington, 1995]. It has been suggested that two sexually transmitted DNA viruses, Epstein-Barr virus (EBV) and human cytomegalovirus (CMV), are possible agents in the development of cervical cancer [Cheung et al., 1993; Shen et al., 1993].

Epstein-Barr virus (EBV) is a human herpes virus

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which is clearly associated with Burkitt's lymphoma and nasopharyngeal carcinoma. More recently, EBV has also been associated with some tonsil, tongue, laryngeal and parotid carcinomas, but the evidence is not conclusive. In addition, EBV has been demonstrated in normal, premalignant and malignant lesions of the uterine cervix, and its possible role in cervical cancer has been suggested [Landers et al., 1993]. CMV has oncogenic potential [Huang et al., 1984]. Recent epidemiological studies have documented cervical shedding of CMV in sexually active women (Collier et al., 1995; Shen et al., 1994]. It has been suggested that the immediate-early (IE) gene products of CMV can transactivate other viral or cellular genes [Boldogh et al., 1991; Colberg-Poley et al., 1991] implying that CMV might play a role, either directly or indirectly, in the pathogenesis of cervical carcinoma.

To date studies have investigated the presence of HPV, CMV and EBV in cervical lesions, ranging from cervicitis to carcinoma [Koffa et al., 1995], but the prevalence of these viruses in the healthy female population has not been studied.

The aim of the present study was to investigate the presence of HPV, CMV and EBV genome in cervical cells from 143 healthy Italian women using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Study Population and Sample Collection

The study group consisted of 143 healthy women (age range 17–70 years, mean age 37.7) who attended the Centre for Preventive Oncology of the University of Rome "La Sapienza" for routine gynaecological examination. Criteria for inclusion in the study were normal cervical smears, absence of apparent inflammatory disease conditions and absence of pregnancy. The women selected were then divided into four groups with respect to age as follows: group I (range 17–25 years, mean age 20.3) n = 48 women; group II (range 26–35 years, mean age 31), n = 30 women; group III (range 36–50 years, mean age 41.8), 32 women; group IV (range 51–70 years, mean age 57.8), n = 33 women. All the subjects gave informed consent.

Cervical cells collected by use of an Ayre spatula were placed immediately in phosphate-buffered saline (PBS) containing penicillin G (100 U/ml), streptomycin (100 μg/ml) and centrifuged at 2000 rpm for 10 min at 4°C. After centrifugation the cellular pellet was resuspended in 100 μl of buffer containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 0.5% Tween 20, 100 μg/ml of Proteinase K. The mixture was incubated at 55°C for 1 hr, then at 90°C for 5 min and stored at −20°C until use. Ten microliters of cell lysate was used for each reaction of PCR amplification.

PCR Amplification

For each sample four different reactions were prepared to detect HPV 16, HPV 18, CMV and EBV genome. PCR was carried out in a final volume of 100 μl consisting of a 10 μl DNA sample, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl (pH 8.3), 200 mM of each dNTP, 2.5 U Taq DNA polymerase (Polymed) and 50–100 pmol of both primers for the specific reaction. Sequences of primers and probes were: HPV 16 sense primer, 5′ ACCGAAACCGGTTAGTATAAAAGC 3′, nucleotides no. 50–73; HPV 16 antisense primer, 5′ GATCAGTTGTCTCTGGTTGCAAAT 3′, nucleotides no. 602–625; HPV 16 probe, 5′ AGCTGCAAACAACTATACATGATATAATATTAGAATGTGT 3′, nucleotides no. 156–195; HPV 18 sense primer, 5′ CACACCACAATACTATGGCGCGCT 3′, nucleotides no. 91–114; HPV 18 antisense primer, 5′ CTGCTGGATTCAACGGTTTCTGGC 3′, nucleotides no. 427–450; HPV 18 probe, 5′ AACTGAACACTTCACTGCAAGACATAGAAATAACCTGTGT 3′, nucleotides no. 136–202; CMV sense primer, 5′ TCCTCCTGCAGTTCGGCTTC 3′, nucleotides no. 531–550; CMV antisense primer, 5′ TTTCATGATATTGCGCACCT 3′, nucleotides no. 751–770; CMV probe, 5′ TGCTGAGCTGCGGCCATCAGA 3′, nucleotides no. 661–680; EBV sense primer, 5′ AGGGGGGACTTTATGTGACC 3′, nucleotides no. 13675–13694; EBV antisense primer, 5′ AATCACTACCAGAGATTACCTG 3′, nucleotides no. 13990–14011; EBV probe, 5′ GCTCTGGAGGCACCTACTCGAGGCAGGCTT 3′, nucleotides no. 13719–13764. The expected sizes of amplification products of HPV 16, HPV 18, CMV and EBV were 576, 360, 239 and 337 bp respectively.

PCR was run for 30 cycles of amplification in a Perkin Elmer-Cetus Thermal Cycler 480. For HPV 16 and HPV 18 amplifications, the PCR cycles were denaturation step at 94°C for 1.5 min, annealing step at 55°C for 2 min and primer extension step at 72°C for 2 min. For CMV amplifications, the PCR cycles were denaturation step at 94°C for 1.5 min, annealing step at 50°C for 2 min and primer extension step at 72°C for 2 min. The PCR amplification conditions used for EBV were denaturation step at 94°C for 1.5 min, annealing step at 57°C for 2 min and finally the extension step at 72°C for 2 min. The final extension steps were always prolonged for another 7 min. Positive controls in the PCR included DNAs from CaSki cell line (for HPV 16), HeLa cell line (for HPV 18), AD 169 infected fibroblasts (for CMV), and Raji cell line (for EBV). In each PCR experiment, we included a sample without DNA as a control. All the recommended precautions were taken to avoid the possibility of false-positive results and the preparation of reaction mixture and analysis of amplified products were carried out in separate rooms.

Analysis of Amplification Products

Twenty microliters of the amplification products was electrophoresed on 2% agarose gel, then the DNA were transferred to the Hybond N membrane (Amersham) and fixed by UV radiation for 5 min. Filters were hybridized with 5′ ^{32}P-end-labeled oligonucleotide probes for 3 hr at 37°C in 6 × SSC, 10 × Denhardt solution, 0.5% sodium dodecyl sulphate (SDS) and 10^6 cpm/ml of appropriate probe. Filters were washed in 1 × SSC, 0.5% SDS at 60°C and autoradiographed on XAR film (Kodak) at −70°C.

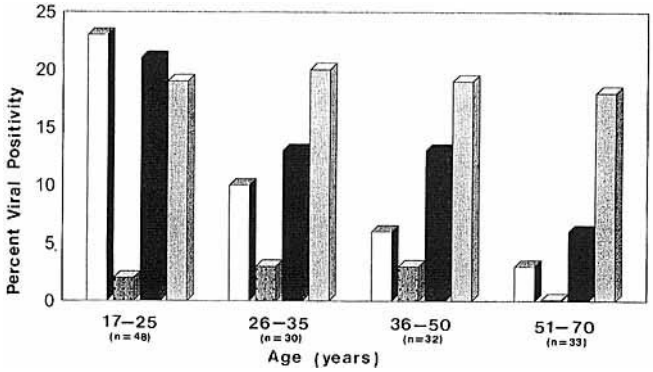

Fig. 1. Relationship between age and detection of HPV, CMV and EBV genomes in normal cervix: ☐ HPV 16; ▩ HPV 18; ■ CMV; ▩ EBV. Group I (aged 17 to 25 years), n = 48 women; group II (aged 26 to 35 years), n = 30; group III (aged 36 to 50 years), n = 32; group IV (aged 51 to 70 years), n = 33.

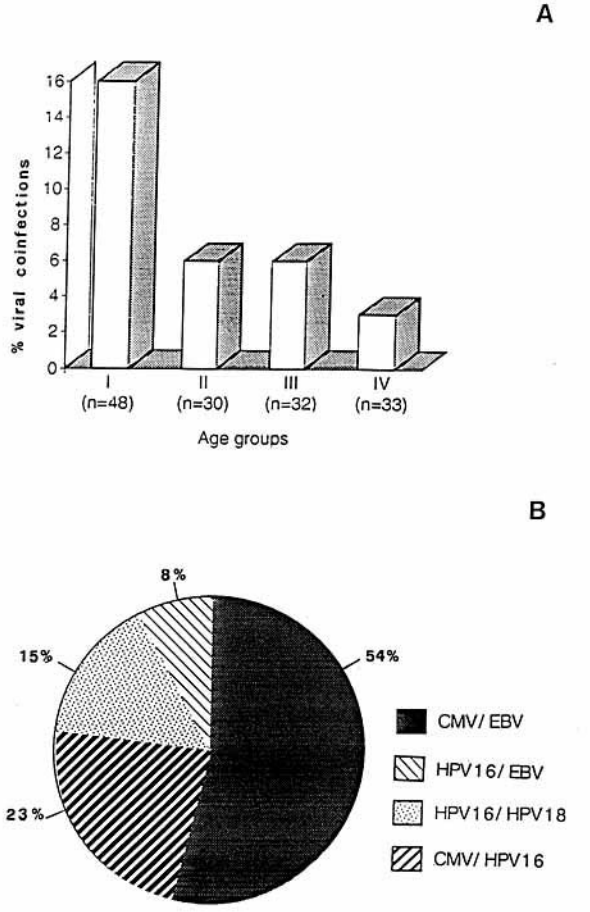

Fig. 2. Prevalence of viral coinfections in normal cervix. **A:** Relationship between age and viral coinfections. **B:** Viral associations in double infections.

RESULTS

Prior to investigation for the presence of HPV, CMV and EBV genomic sequences by PCR, all specimens were analyzed with β-globin primers to assess DNA accessibility [Saiki et al., 1988]. All samples were found suitable. The PCR conditions, which were different for each virus, had similar sensitivity for each system used, so that each virus was detected with the same sensitivity. After amplification by PCR and Southern blot analysis of the amplified products, 53 of 143 samples (37%) were found positive for the presence of viral genome, while viral DNA was not identified in the remaining 90 samples. In order to analyse the prevalence of viral infections with respect to age, the study population was divided into four groups as follows: group I (17 to 25 years); group II (26 to 35 years); group III (36 to 50 years); group IV (51 to 70 years). In the first age group the percentage of positivity was 47.9% (23/48), in the second group it was 40% (12/30), in the third, 31.25% (10/32), and in the fourth, 24.2% (8/33). The percentage of HPV 16 DNA positive samples was 23% in the first group, 10% in the second, 6% in the third and 3% in the fourth. HPV 18 DNA was detected only in the first three groups, without significant difference among the three groups with percentages of 2% in the first group and 3% in the second and third age group respectively.

Analysis of CMV amplification products produced by PCR using primers from the CMV specific IE2 region shows that the percentage of infection among the different groups was 21% in the first, 13% in the second and third groups and 6% in the fourth age group.

Finally, EBV DNA was identified in each age group as follows: 19% in the first and third groups, 20% in the second, and 18% in the fourth group. The percentages of virus infection for each group are shown in Figure 1.

Coinfections were found in each group: 16% in the first, 6% in the second and third, and 3% in the fourth group (Fig. 2A). We detected the following viral double infections: CMV/EBV (53.84%); CMV/HPV 16 (23%); HPV16/HPV18 (15.38%); HPV16/EVB (7.69%) (Fig. 2B).

DISCUSSION

Considerable experimental and epidemiological evidence has been collected indicating the important role of human papillomaviruses (HPVs), mainly HVP 16, in the pathogenesis of squamous cell carcinomas of the cervix. More recently, the role of two other sexually transmitted virus, CMV and EBV, have been under investigation in cervical cancer [Shen et al., 1993; Wong et al., 1993; van den Brule et al., 1995]. It is assumed that in HVP 16 infected healthy women, cervical cancer can be induced after a long latency period and, therefore, previous infections would be an explanation for its development. Our results indicate that the infection rates of HPV 16 decrease with age ranging from 23% in the first age group (17–25 years) to 6% in the last (51–70 years) ($P = 0.023$).

Likewise, there are decreased rates of infection with cytomegalovirus correlated to age, 14% infection among the examined population ranging from 21% in the group of younger women to 6% in older women. EBV infection

rates do not appear to decrease with increasing age. The results also demonstrate that HPV 18 is rarely present (0–3%) in the cervix of healthy women, independently of age.

It is clear from the results that HPV 16 and CMV are prevalent among younger women, probably due to greater freedom of sexual activity. Taken together, these results show a significantly higher prevalence (48%) of sexually transmitted viruses in the first age group with respect to the fourth group ($P = 0.05$). Furthermore, in the first age group a higher percentage of double infections (16.6%) was found while the percentage of double infections in the second and third groups was 6%, and 3% in the fourth group. HPV 16-CMV coinfections were found as well as HPV 16-EBV, CMV-EBV and HPV 16–18.

Whether the presence of EBV is relevant or indeed capable of interacting with HPVs or other viruses, such as CMV, has yet to be established. However, EBV and CMV are undoubtedly good candidates in a multiviral hypothesis for carcinogenesis. Our results show that the cervix frequently harbours EBV, CMV and HPV, and may suggest the possible role of some synergistic effect or interaction between these viruses.

Epidemiological studies have shown that the risk of developing cervical cancer is increased by several factors, including viral infection, cigarette smoking, the use of oral contraceptives, early age of first sexual intercourse, and multiple sexual partners. Furthermore, epidemiological studies have shown that the onset of sexual activity at early and sexual promiscuity are also risk factors for the development of cervical cancer, possibly related to the prevalence of HPV. Clearly, long-term prospective studies are needed to establish the implications of viral infections as well as coinfections in asymptomatic women, and the role of virus associations in the development of cervical cancer.

The accurate determination of HPV, CMV and EBV prevalence in normal and neoplastic cervical cells will contribute to the understanding of the natural history of these viruses and the role of each virus in cervical diseases.

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